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14. ABSTRACT

Prostate cancer (PCa) is the most commonly diagnosed cancer in men and is the second leading cause of cancer death in the USA. Most prostate cancer patients are treatable, but the patients usually die due to drug resistance and metastatic disease. Hypoxia, a common characteristic in solid tumors including PCa, with deregulated expression of hypoxia-inducible factors (HIF) and its biological consequence lead to poor prognosis of patients diagnosed with solid tumors, resulting in higher mortality. Cancer stem cells (CSCs) are reportedly associated with therapeutic resistance and contribute to aggressive tumor phenotypes. miRNAs, critical regulators for gene expression, have an important role in tumorigenesis. A large number of miRNAs have been reported to be responsive to hypoxia and HIF pathway in a wide range of cells and tissues including cancer cells. In this study, we demonstrate that hypoxia increases cell migration, invasion, angiogenesis, prostatosphere formation, the productions of VEGF and IL-6, the expression of CSC genes Nanog, Oct4, EZH2 as well as miR-21 and miR-210, oncogenic miRNAs, in human PCa cells. Treatment with CDF, a novel Curcumin-derived analog previously showing an anti-tumor effect in vivo, inhibits the productions of VEGF and IL-6, and the expression of Nanog, Oct4, EZH2 mRNAs, as well as miR-21 and miR-210 in these cells under hypoxia. CDF also decreases cell migration, invasion, angiogenesis, prostatosphere formation in prostate cancer cells under hypoxia. Taken together, these data indicates the anti-tumor effect of CDF may be, in part, associated with its inhibition of tumor hypoxic pathways by targeting hypoxia-mediated miRNAs.

15. SUBJECT TERMS
Hypoxia, cancer stem cells, miRNAs, curcumin-derived analog CDF, prostate cancer

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Introduction

Prostate Cancer (PCa) is the most commonly diagnosed cancer in men and is the second leading cause of cancer death in the USA (1). Most PCa patients are treatable, but the patients usually die due to drug resistance and metastatic disease. Thus, there is a dire need for the development of novel strategies by which drug resistance and metastatic disease could be controlled with novel agents with better treatment outcome. Hypoxia is one of the fundamental biological phenomena that are intricately associated with the development and aggressiveness of a variety of solid tumors including PCa. Hypoxia-inducible factors (HIF) function as a master transcription factor, which regulates hypoxia responsive genes and have been recognized to play critical roles in tumor invasion, metastasis, and chemo-radiation resistance, and contributes to increased cell proliferation, survival, angiogenesis and metastasis (2, 3). Therefore, tumor hypoxia with deregulated expression of HIF and its biological consequence lead to poor prognosis of patients diagnosed with solid tumors, resulting in higher mortality, suggesting that understanding of the molecular relationship of hypoxia with other cellular features of tumor aggressiveness would be invaluable for developing newer targeted therapy for solid tumors. It has been well recognized that cancer stem cells (CSCs) and epithelial-to-mesenchymal transition (EMT) phenotypic cells are associated with therapeutic resistance and contributes to aggressive tumor growth, invasion, metastasis, and are believed to be the cause of tumor recurrence (4). Emerging evidence suggest that hypoxia and HIF pathway enhance the phenotypes and functions of CSC and EMT (5-9), contributing to tumor aggressiveness, which could also be due to deregulation of microRNAs (miRNAs).

The miRNAs are known to play critical roles in a wide array of biological processes, including cell differentiation, proliferation, death, metabolism and energy homeostasis (10, 11). Accumulating evidence has suggested that miRNAs might have an important role in the development and progression of tumors. The altered expression of miRNAs has been associated with clinical prognosis of tumor, resistance to chemo-radiation therapy, tumor recurrence and or relapse. A large number of miRNAs have been reported to be responsive to hypoxia and HIF pathway in a wide range of cells and tissues including cancer cells (12-15). It has been reported that hypoxia causes decreased expression of miR-101, a potential anti-oncogenic miRNA, and increased expression of miR-21 and miR-210, oncogenic miRNAs in various cancers including PCa (16, 17). Thus, hypoxia-mediated regulation of miRNAs may play important roles in tumor aggressiveness mediated through the regulation of cellular signaling pathways including HIF pathway. Targeting these hypoxia-mediated miRNAs might provide a novel therapeutic strategy for the prevention and/or treatment of PCa. Here, we have examined the effect of hypoxia on cell migration, invasion, angiogenesis, VEGF, IL-6, CSC genes, miR-21, and miR-210 in PCa cells under hypoxic condition as proposed in the grant application. We also investigated the role of miR-21 in the regulation of VEGF, IL-6, the formation of prostaspheres, and CSC signature genes in PCa cells. Furthermore, we examined the effect of a novel curcumin-derived analogue (CDF) that showed anti-tumor activity with a greater systemic and target tissue bioavailability, and investigated the effects of CDF in cell survival, migration, invasion, angiogenesis, formation of prostaspheres, and the expression of HIF-1α, VEGF, IL-6, CSC signature genes, and miRNAs in PCa cells under hypoxic condition. **The results of our investigation are presented below.**

Body of the report

Following are the specific aims that were proposed in the grant application.

In aim-1, we will investigate the expression of HIF-1α and its target gene, VEGF, and assess the expression of miR-200 family, EMT biomarkers (E-cadherin and γ-catenin as epithelial markers; vimentin, ZEB1, N-cadherin as mesenchymal markers), and CSC biomarkers (CD44, CD133, and EpCAM) in PCa cells under hypoxic conditions.

In aim-2, we will investigate the molecular role of HIF-1α and miR-200 in the acquisition of EMT phenotype, the expression of CSC markers, and the capacity of CSC self-renewal under hypoxic condition of PCa cells using under- and over-expression techniques (HIF-1α specific inhibitors, siRNAs, cDNA expression vectors).

In aim-3, we will assess the effect of CDF on cell survival, apoptosis, clonogenicity, invasion, EMT markers, CSC markers, miR-200, and HIF-1α in human PCa cells under hypoxic condition.

The summary of our observations:

1. Effect of CDF on cell survival of PCa cells under hypoxic condition. In order to investigate the effect of CDF on cell survival in human PCa cells under hypoxic condition, MTT assay was conducted using human PCa PC-3 and LNCaP cells. Hypoxic (1% O_2) and 5% CO_2 conditions were generated by the control of input flow rates of nitrogen and carbon dioxide, respectively. 3000 cells were plated each well of the 96-well plates and incubated at standard culture conditions either normoxic condition (21% O_2) or hypoxic condition (1% and 5% CO_2) overnight. The cells were treated with different concentrations of CDF (0-1.5 μM) and incubated at 8h of hypoxic condition followed by 16h of normoxic condition each day. After 3 days of treatment, the cells were harvested for MTT assay, as described in our previous publication (18, 19). The results indicate that CDF remarkably inhibited cell survival of PC-3 and LNCaP cells in a dose-dependent manner **(Figure 1).**

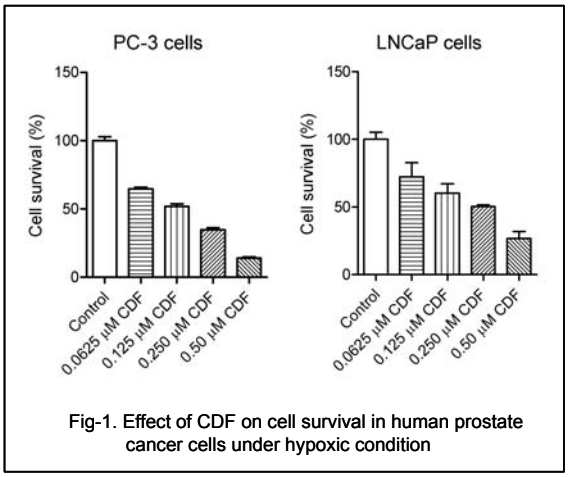

Fig-1. Effect of CDF on cell survival in human prostate cancer cells under hypoxic condition

2. Effect of CDF on clonogenicity of PCa cells under hypoxic condition. Clonogenic assay was conducted to examine the effect of CDF (0.5 μmol/L) on cell growth and proliferation of PCa cells as described previously (18, 19). Briefly, 5 x10^4 cells were plated in a six-well plate and after 72h of exposure to 0.5 μmol/L of CDF, the cells were trypsinized and 1,000 single viable cells were plated in 100-mm Petri dishes. The cells were then incubated for 10 to 12 days at 37°C in a 5% CO_2/5% O_2/90% N_2 incubator. Colonies were stained with 2% crystal violet, washed with water, and counted. The results showed the CDF treatment on clonogenicity of PC-3 and LNCaP cells under hypoxic condition **(Figure 2**).

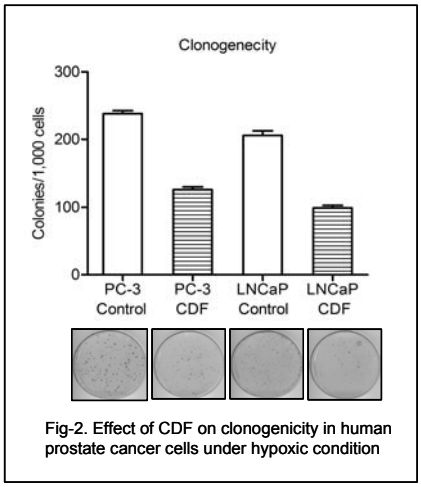

Fig-2. Effect of CDF on clonogenicity in human prostate cancer cells under hypoxic condition

3. Effect of CDF on the expression of HIF-1α protein in PCa cells under hypoxic condition. In order to examine the effects of CDF on the expression of HIF-1α protein in PCa cells under hypoxic condition, we measured the relative level of HIF-1α protein by Western blot analysis using whole cell protein lysates of PCa cells. Total cell lysates of the cells exposed to 16h of hypoxic condition were obtained by lysing the cells in protein lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 2 mM sodium fluoride, 2 mM Na_3VO4_2, 1 mM EDTA, 1 mM EGTA, and 1 x protease inhibitor cocktail, and Western blotting was performed as described previously (18, 19). As

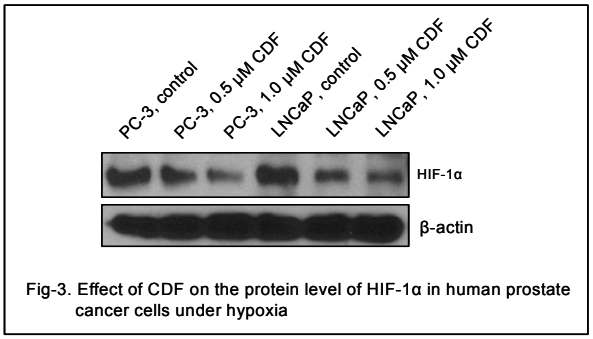

Fig-3. Effect of CDF on the protein level of HIF-1α in human prostate cancer cells under hypoxia

shown in **Figure 3 above,** CDF treatment decreased the relative level of HIF-1α in PC-3 and LNCaP cells under hypoxic condition.

4. Effect of CDF on VEGF production in PCa cells under hypoxic condition.

In order to examine the effect of CDF on hypoxia-induced VEGF production in PCa cells, we harvested the cultured media from PC-3 cells and LNCaP cells under normoxic and hypoxic conditions for 16h and conducted ELISA assay (R&D Systems), following the manufacturer's manual. The results show that the cells incubated at hypoxic condition increased VEGF production, compared to the cells incubated at normoxic condition **(Figure 4).** CDF treatment remarkably decreased the production of hypoxia-induced VEGF in PCa cells **(Figure 4).** HIF-1α over-expressing PC-3 cells increased VEGF production under hypoxic condition, compared to its parental PC-3 cells. CDF treatment also inhibited the hypoxia-induced VEGF production in HIF-1α over-expressing PC-3 cells.

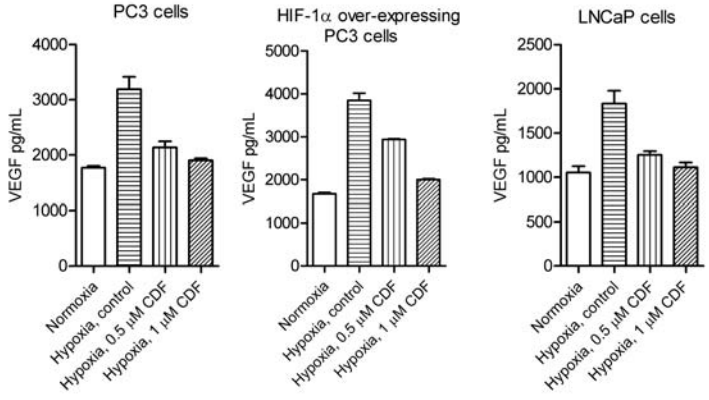

Fig-4. Effect of CDF on VEGF production in human prostate cancer cells under hypoxic condition

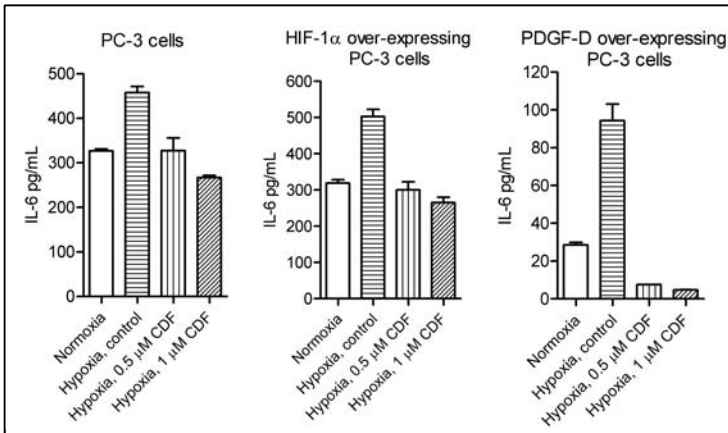

Fig-5. Effect of CDF on the production of inflammatory cytokine IL-6 in human prostate cancer cells under hypoxic condition

5. Effect of CDF on inflammatory cytokine IL-6 production in PCa cells under hypoxic condition. Large numbers of studies have indicated that inflammatory cytokine IL-6 plays an important role in the development and progression of tumors including PCa. Emerging evidence suggests that hypoxia induces the expression of inflammatory cytokines including IL-6 in a wide range of cells including cancer cells. IL-6 has been shown to be a direct regulator of the CSC self-renewal capacity (20, 21). In order to investigate the effect of CDF on IL-6 production in PCa cells under hypoxic condition, we measured the levels of IL-6 in the culture media under normoxic and hypoxic conditions by ELISA assay. The results suggest that the cells incubated at hypoxic condition showed increased IL-6 production, compared to the cells incubated under normoxic condition **(Figure 5).** CDF treatment remarkably decreased the production of hypoxia-induced IL-6 in PCa cells **(Figure 5).** CDF also decreased hypoxia-induced IL-6 production in HIF-1α over-expressing and PDGF-D over-expressing PC-3 cells **(Figure 5).**

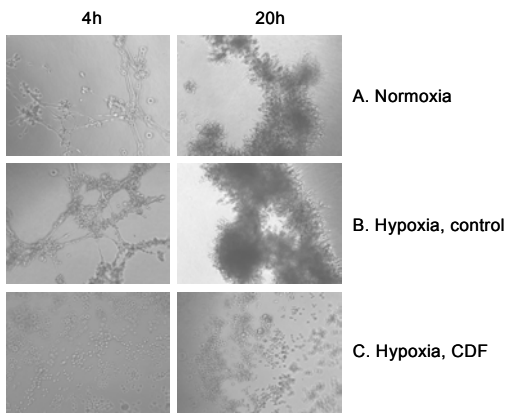

Fig-6. Effect of CDF on angiogenesis in vitro in vascular endothelia cells under hypoxic condition

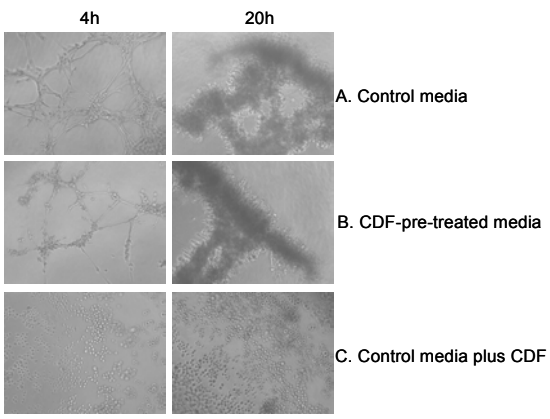

Fig-7. Effect of CDF on angiogenesis in vitro in vascular endothelia cells under hypoxic condition

6. Effect of CDF on angiogenesis *in vitro* in vascular endothelial cells under hypoxic condition. In order to examine the effect of CDF on angiogenesis *in vitro* in vascular endothelial cells under hypoxic condition, we conducted tube formation assay, as described previously (22, 23). Briefly, $3x10^4$ vascular endothelial cells were plated each well of the Matrigel-coated 96-well plate in 100 μL of 10% FBS-DMEM medium, and exposed to normoxic or hypoxic conditions for 4h of incubation at 37°C, followed by 16h of normoxic condition. The photograph was taken at 4h and 20h, respectively. The results show that hypoxic condition increased the tube formation in vascular endothelial cells at 4h and 20h of incubations, respectively, compared to normoxic condition. CDF treatment inhibited the hypoxia-induced tube formation in vascular endothelial cells **(Figure 6; please see previous page).** To clarify whether or not CDF-mediated molecules or CDF itself contributes to the inhibition of tube formation, we collected non-CDF-treated (control) and CDF-treated condition media from cancer cells and conducted the tube formation assay under normoxic condition. We found that the vascular endothelial cells incubated with control condition media had increased tube formation at 4h and 20h, compared to the cells incubated with CDF-pre-treated condition media. Addition of CDF to the control condition media significantly inhibited the tube formation, compared to the cells incubated at control condition media and CDF-pre-treated condition media **(Figure 7; please see the previous page).** These data suggest that CDF itself contributes to the inhibition of the tube formation.

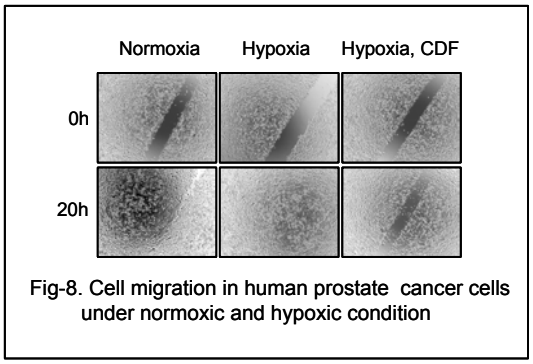

Fig-8. Cell migration in human prostate cancer cells under normoxic and hypoxic condition

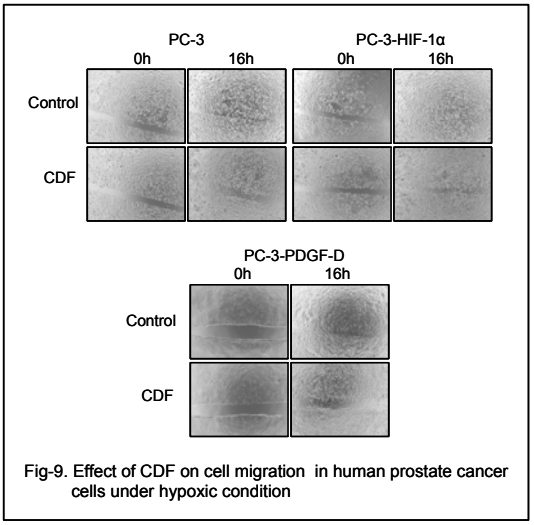

Fig-9. Effect of CDF on cell migration in human prostate cancer cells under hypoxic condition

7. Effect of CDF on cell migration *in vitro* in PCa cells under hypoxic condition. In order to examine the effect of CDF on cell migration of PCa cells under hypoxic condition, we conducted wound healing assay, as described previously. Briefly, when the PC-3 cells reached 90-95% confluent, the wound was generated by scratching the surface of the plates with a pipette tip. The cells were then incubated in the absence and presence of CDF (0.5 μmol/L) and were cultured under hypoxic condition for 4h, followed by 16h of normoxic condition, and then photographed with a Nikon Eclipse TS100 microscope, as described previously (24, 25). The results show that hypoxia-exposed PC-3 cells had increased capacity of wound healing, compared to the cells cultured under normoxia **(Figure 8).** CDF treatment inhibited the wound healing capacity in cancer cells under hypoxic condition **(Figure 8).** We also examined the effect of CDF on cell migration in HIF-1α over-expressing and PDGF-D over-expressing PC-3 cells under hypoxic condition by wound healing assay. As shown in **Figure 9,** we found that over-expression of HIF-1α and PDGF-D increased the wound healing capacity in PC-3 cells exposed to 16h of hypoxic condition. CDF treatment inhibited the wound healing capacity in both HIF-1α-over-expressing and PDGF-D over-expressing PC-3 cells under hypoxic condition **(Figure 9).** These results provided convincing data showing that CDF inhibits hypoxia-induced cell migration of PCa cells, even in HIF-1α-over-expressing and PDGF-D-over-expressing PCa cells.

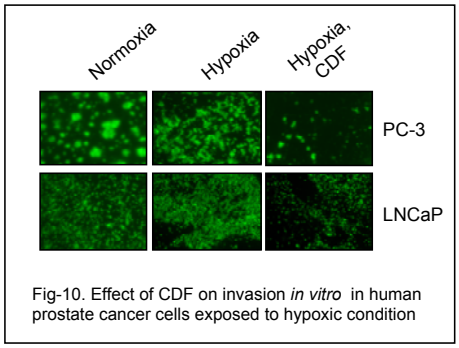

Fig-10. Effect of CDF on invasion *in vitro* in human prostate cancer cells exposed to hypoxic condition

8. Effect of CDF on cell invasion *in vitro* in PCa cells exposed to hypoxic condition. In order to examine the effect of CDF on invasion of PCa cells under hypoxic condition, we conducted *in vitro* invasion assay by using Costar Transwell 24-well-plates with polycarbonate membrane (Corning Incorporated, Corning, NY), as described previously (19). Briefly, $4x10^4$ of cancer cells (PC-3 and LNCaP) exposed to 3 days of incubation

under normoxic or hypoxic condition were seeded in each well of the Matrigel pre-coated Transwell plates, and incubated at standard culture condition. After 20h of incubation either in the absence or presence of CDF (1 μM), the invaded cancer cells were stained with calcein-AM (Invitrogen) in PBS solution, following the manufacturer's manual. The photographs were taken using a fluorescent microscope. As shown in **Figure 10 (please see previous page),** both PC-3 and LNCaP cells exposed to hypoxic condition had increased capacity of invasion, compared to those cells exposed to normoxic condition. CDF treatment inhibited the capacity of hypoxia-induced invasion of PCa cells.

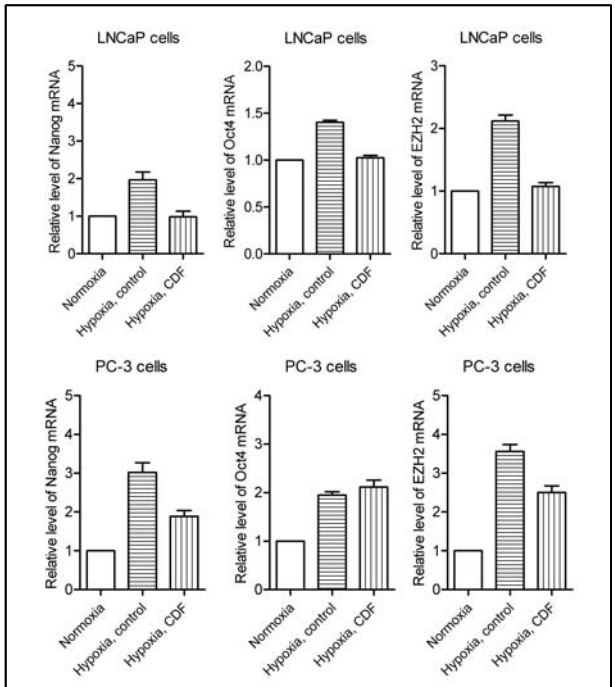

Fig-11. Effect of CDF on the expression of Nanog, Oct4, and EZH2 mRNAs in human prostate cancer cells under hypoxic condition.

9. Effect of CDF on the gene expression of cancer stem cell (CSC) markers in PCa cells under hypoxic condition. In order to examine the effect of CDF on cancer stem cell (CSC) gene markers Nanog, Oct4, and EZH2 in PCa cells under hypoxic condition, we measured the relative levels of Nanog, Oct4, and EZH2 mRNAs in PC-3 and LNCaP cell under hypoxic condition by real-time RT-PCR. To determine the mRNA expression, two micrograms of total RNAs extracted from each sample were used for RT reaction in 20 μL of reaction volume using a reverse transcription system (Invitrogen) according to the manufacturer's instruction. SYBR Green Assay kit (Applied Biosystems, Carlsbad, CA) was used for real time PCR reaction, following manufacturer's protocol. Sequences of PCR primers were described previously (26). Data were analyzed using C_t method and were normalized by GAPDH expression in each sample. As shown in **Figure 11,** hypoxia induced the relative mRNA levels of Nanog, Oct4, and EZH2 in PC-3 and LNCaP cells whereas CDF decreased the levels of Nanog, Oct4, and EZH2 mRNAs in PCa cells under hypoxic condition.

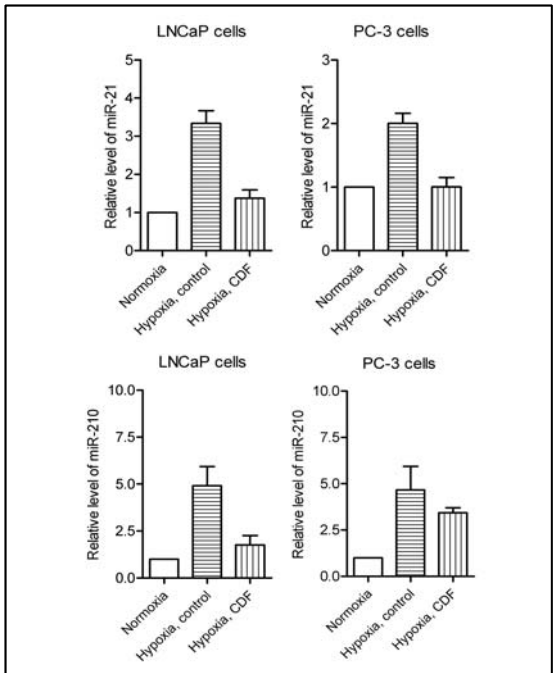

Fig-12. Effect of CDF on the miRNA expression of miR-21 and miR-210 in human prostate cancer cells under hypoxia.

10. Effect of CDF on the miRNA expression of miR-21 and miR-210 in PCa cells under hypoxic condition. It has been reported that hypoxic conditions induces the expression of miR-21 and miR-210 in various cells including cancer cells (16, 17). In order to examine the effect of CDF on miRNA expression in PCa cells under hypoxic condition, we measured the relative levels of miR-21 and miR-210 under hypoxic condition by real-time RT-PCR by using TaqMan MicroRNA Assay kit (Applied Biosystems) following manufacturer's protocol. 5 ng of total RNA was reverse transcribed and real-time PCR reactions were carried out in 10 μL of reaction mixture as described previously (26, 27) using AB StepOnePlus Real-Time PCR System (Applied Biosystems). Data were analyzed using C_t method and were normalized by RNU48 expression in each sample. As shown in **Figure 12**, hypoxia induced the relative miRNA levels of miR-21 and miR-210 in PC-3 and LNCaP cells whereas CDF decreased the levels of miR-21 and miR-210 mRNAs in PCa cells under hypoxic condition.

11. Effect of CDF or anti-miR-21 on the self-renewal capacity of CSC-like cells in human PCa cells under hypoxic condition. In order to assess the effect CDF or anti-miR-21 on the self-renewal capacity of CSC-like cells in human PCa cells under hypoxic condition, we conducted the sphere formation assay using human PCa PC-3 and LNCaP cells, as described previously (14). Briefly, single cell suspensions of cells were plated on ultra low adherent wells of 6-well plate (Corning, Lowell, MA) at 1,000 cells/well in sphere formation medium (1:1 DMEM/F12 medium supplemented with B-27 and N-2 (Invitrogen), and exposed to hypoxic condition every other day. After 7 days, the spheres termed as "prostaspheres" were collected by centrifugation (300xg, 5 min), and counted. The proportion of sphere-generating cells was calculated by dividing the number of prostaspheres by the number of seeded cells with the diameter greater than 50 µmeters. As shown in Figure 13, the cells exposed to hypoxic condition had increased numbers of prostaspheres, compared to the cells exposed to normoxic condition. CDF treatment decreased the formation of prostaspheres in a dose-dependent manner. We also investigated the role of miR-21 in the regulation of the formation of prostaspheres in PC-3 cells by transfection of anti-miR-21 to the cells. Briefly, 1000 PC-3 cells were seeded each well in the low attachment 24-well plates (Corning) in sphere formation medium and transfected with anti-miR-21 (Ambion, Austin, TX) using DharmaFECT transfection reagent (Dharmacon), following the manufacturer's protocol, and as described previously (19). The results show that anti-miR-21 decreased the formation of prostaspheres in PC-3 cells **(Figure 13).** These data suggest that miR-21 may play an important role in the regulation of the self-renewal capacity of CSC-like PCa cells.

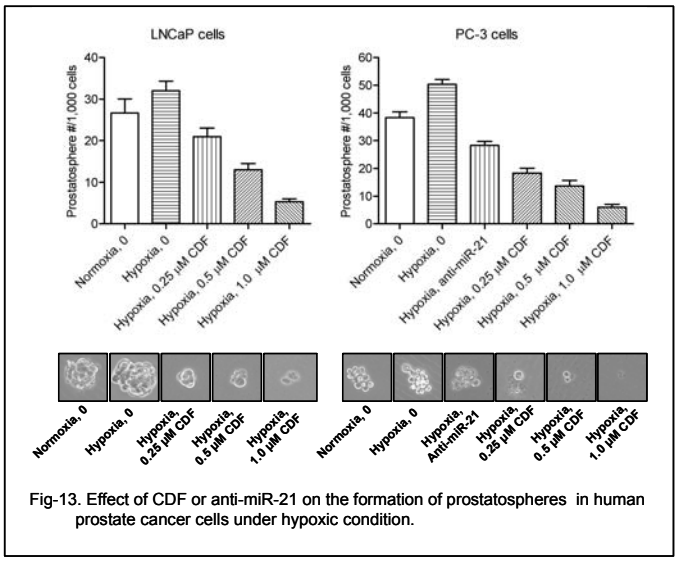

Fig-13. Effect of CDF or anti-miR-21 on the formation of prostatospheres in human prostate cancer cells under hypoxic condition.

12. Effect of CDF or anti-miR-21 on the expression of CSC surface marker proteins CD44 and EpCAM in PC-3 sphere cells under hypoxic condition. In order to examine whether or not CDF or anti-miR-21 inhibits the expression of CSC surface markers CD44 and EpCAM in CSC-like cells of PCa, we conducted confocal imaging microscopy of CD44 and EpCAM in the sphere forming cells of PC-3 cells. 10,000 PC-3 cells were seeded in the sphere formation medium as described above, and either treated with CDF (0.5 µM) or transfected with anti-miR-21, as described above, followed by culturing under hypoxic condition every other days. After 7 days of incubation, the prostaspheres were collected by low speed of centrifugation, washed with 1xPBS, and fixed with 3.7% paraformaldehyde. Monoclonal CD44 and EpCAM antibodies (Cell Signaling) were used for immuno-staining following the manufacturer's protocol as described previously (19). The CD44 or EpCAM-labeled pancreaspheres were photographed under a Nikon ESLIPSE E800. Confocal microscope (Leica TCS SP5) available at the MIRL Core Facility of Wayne State University School of Medicine. The results show that anti-miR-21 decreased the expression of CD44 and EpCAM in PC-3 sphere forming cells under hypoxic condition,

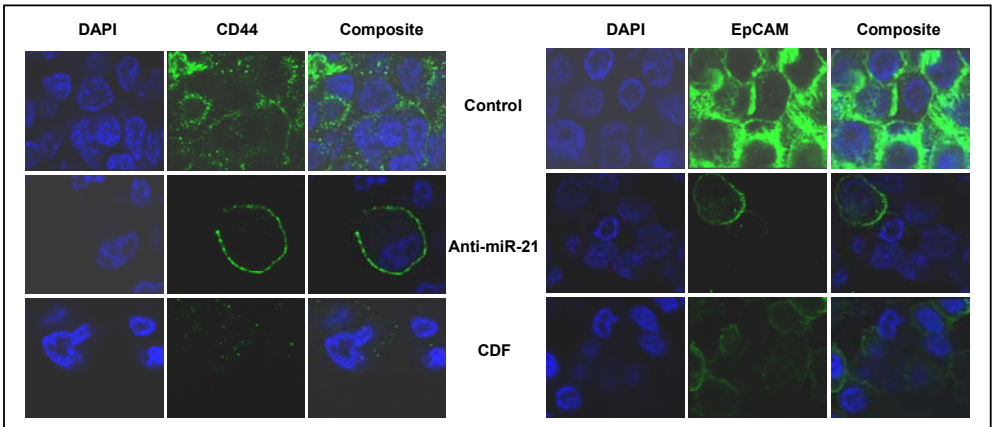

Fig-14. Effect of CDF or anti-miR-21 on CSC surface markers CD44 and EpCAM in the sphere cells of human prostate cancer PC-3 cells under hypoxic condition.

consistent with the CDF treatment **(Figure 14).** These data suggest that CDF down-regulates the formation of prostaspheres and the expression of CD44 and EpCAM by potentially targeting the expression of miR-21.

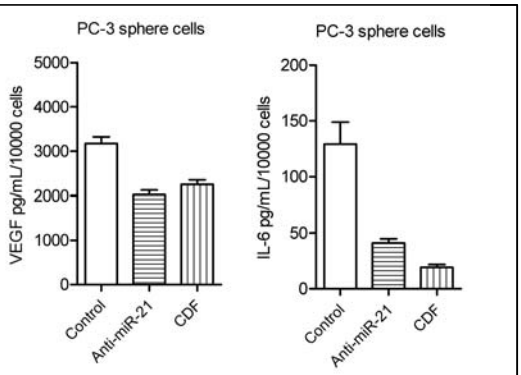

Fig-15. Effect of CDF or anti-miR-21 on VEGF and IL-6 production in the PC-3 sphere cells of human prostate cancer cells under hypoxic condition.

13. Effect of CDF or anti-miR-21 on VEGF and IL-6 production in the sphere cells of PC-3 cells under hypoxic condition. We examined the effect of CDF on VEGF and IL-6 productions in the sphere forming cells of PC-3 cells under hypoxic condition by ELISA assay. We found that PC-3 sphere forming cells produced a larger amount of VEGF under hypoxic condition, compared to its parental PC-3 cells (3,172 pg/mL/10^4 PC-3 sphere cells vs 3192 pg/mL/10^6 PC-3 cells; **Figure 4 and 15**), suggesting that the PC-3 sphere forming cells may promote angiogenesis by up-regulating the expression of VEGF production. We also found that CDF treatment decreased hypoxia-induced VEGF production in PC-3 sphere forming cells, consistent with the results from the PC-3 sphere forming cells with conditional inhibition of miR-21. Furthermore, we found that similar to VEGF production, the sphere forming cells produced a remarkably higher amount of hypoxia-induced IL-6, compared to its parental cells (129.3 pg/mL/10^4 PC-3 sphere cells vs 457.6 pg/mL/10^6 PC-3 cells; **Figure 5 and 15**). CDF treatment or conditional deficiency of miR-21 by its inhibitor/siRNA decreased the production of hypoxia-induced IL-6 in the sphere forming cells **(Figure 15).**

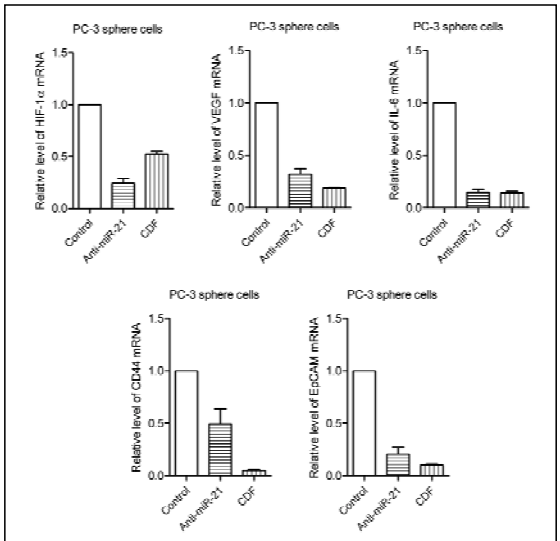

Fig-16. Effect of CDF or anti-miR-21 on the relative levels of HIF-1α, VEGF, IL-6, CD44, and EpCAM mRNAs in the PC-3 sphere cells of human prostate cancer cells under hypoxic condition.

14. Effect of CDF or miR-21 deficiency on the gene expression of HIF-1α, VEGF, IL-6, CD44, and EpCAM in PC-3 sphere cells under hypoxic condition. In order to examine the Effect of CDF or miR-21 deficiency on the gene expression of HIF-1α, VEGF, IL-6, CD44, and EpCAM in CSC-like cells, we measured the relative levels of HIF-1α, VEGF, IL-6, CD44, and EpCAM mRNAs in PC-3 sphere forming cells under hypoxic condition by real-time RT-PCR, as described above. As shown in **Figure 16,** CDF treatment decreased the relative levels of HIF-1α, VEGF, IL-6, CD44, and EpCAM in PC-3 sphere forming cells under hypoxic condition. Similarly, conditional deficiency of miR-21 by anti-miR-21 inhibitor also suppressed these gene expressions in PC-3 sphere forming cells under hypoxic condition **(Figure 16).**

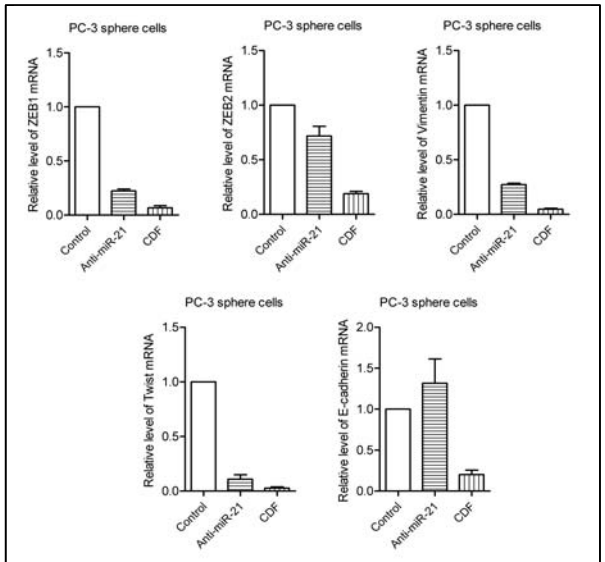

Fig-17. Effect of CDF or anti-miR-21 on the relative mRNA levels of EMT markers in the PC-3 sphere cells of human prostate cancer cells under hypoxic condition.

15. Effect of CDF or miR-21 deficiency on the gene expression of EMT markers in PC-3 sphere cells under hypoxic condition. We also examined whether or not CDF or miR-21 deficiency regulates the gene expression of EMT markers in PC-3 sphere forming cells under hypoxic condition. We found that conditional suppression of miR-21 resulted in a significant decrease in the relative mRNA levels of mesenchymal markers of EMT such as ZEB1, Vimentin, and Twist, and increased the relative mRNA level of epithelial marker E-cadherin in PC-3 sphere cells under hypoxic

condition **(Figure 17).** Similarly, CDF decreased the mRNA levels of mesenchymal markers ZEB1, ZEB2, Vimentin, and Twist in PC-3 sphere forming cells under hypoxic condition. However, CDF also decreased gene expression of E-cadherin in PC-3 sphere forming cells under hypoxic condition **(Figure 17).**

16. Effect of CDF or miR-21 deficiency on miR-21 binding activity to 3'-UTR in PC cells under hypoxic condition by luciferase. In order to examine the effect of CDF or miR-21 deficiency on miR-21 binding activity to 3'-UTR in PC cells under hypoxic condition, we conducted miR-21-mediated luciferase reporter gene assay in PC-3 cells by using miR-21-mediated luciferase reporter gene vector (Signosis, Sunnyvale, CA), in which miR-21 binding to its DNA binding site at the luciferase gene vector suppresses the luciferase activity. Briefly, 10^4 PC-3 cells were seeded in each well of the 96-well plates, and incubated overnight at the standard culture condition. The cells were then transfected with miR-21-suppressed luciferase reporter gene vector (Signosis, Sunnyvale, CA) by using ExGen 500 transfection reagent (Fermentas, Germany) or co-transfected with the luciferase vector and anti-miR-21 by using DharmaFECT transfection reagent (Dharmacon) following the manufacturer's protocol, as described above. After overnight of transfection, the transfectants were treated with CDF for another 20h under standard culture condition, and exposed to 4h of hypoxic condition. Finally, the transfectants were harvested for luciferase assay by using Luciferase Assay System (Promega), following the manufacturer's manual. As shown in **Figure 18,** we found that hypoxia decreased the luciferase activity in the PC-3 cells transfected with miR-21-mediated luciferase vector, compared to the same cells under normoxic condition, suggesting that hypoxia increased the miR-21 binding activity, leading to the decrease in the luciferase activity. Anti-miR-21 increased the luciferase activity in PC-3 cells under hypoxic condition, compared to the same cells without treatment, suggesting that anti-miR-21 decreased the miR-21 DNA binding, leading to an increase in the luciferase activity in PC-3 cells under hypoxic condition. Similarly, CDF treatment increased the luciferase activity in PC-3 cells under hypoxic condition in a dose-dependent manner, suggesting that CDF inhibits the miR-21 DNA binding in PC-3 cells.

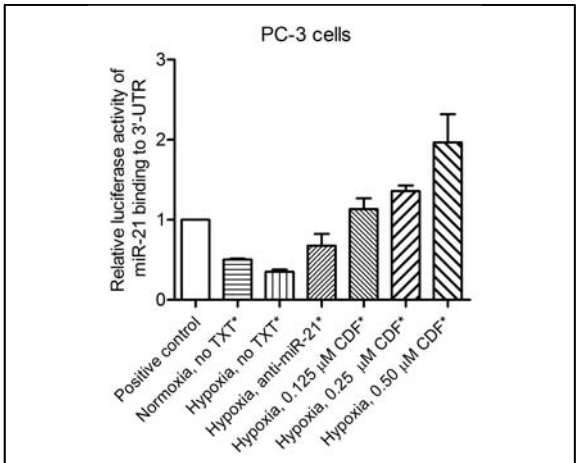

Fig-18. The relative luciferase activity of miR-21 binding to 3'-UTR in PC-3 cells treated with anti-miR-21 and CDF under hypoxia (*: indicated the cells transfected with miR-21-mediated luciferase vector; TXT: treatment).

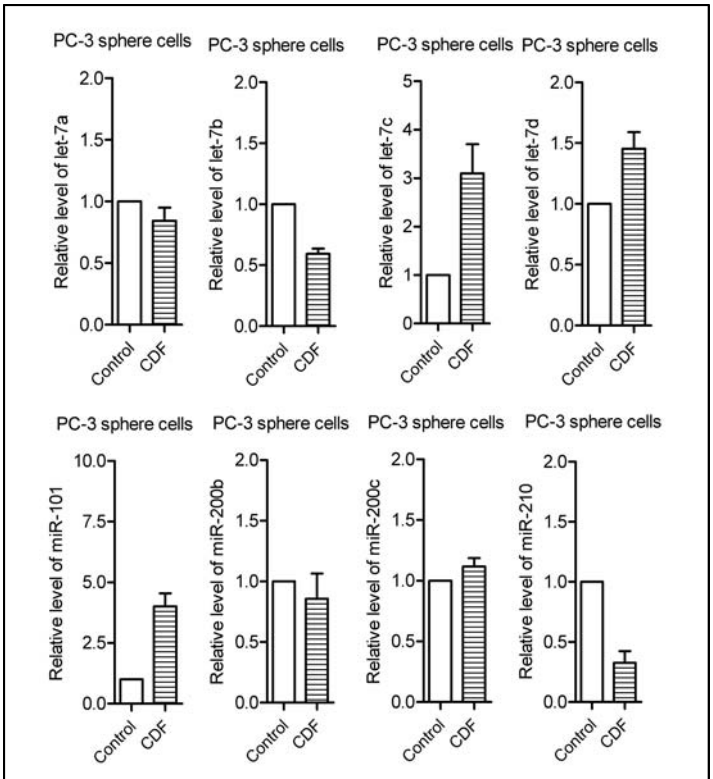

Fig-19. Effect of CDF on the relative levels of miRNAs in the PC-3 sphere cells of human prostate cancer cells under hypoxia.

17. Effect of CDF on the miRNA expression in PC-3 sphere forming cells under hypoxic condition. Recent experiment studies have demonstrated that hypoxia modulates the expression of a number of miRNAs, including oncogenic (let-7 and miR-101) and anti-oncogenic (miR-21 and miR-210) miRNAs (12, 14, 15, 17, 28, 29). In order to further examine the effect of CDF on the expression of these miRNAs, we measured the levels of these miRNA in PC-3 sphere forming cells under hypoxic condition by real-time RT-PCR, as described above. The results show that CDF increased the relative miRNA levels of let-7c,d, and miR-101 and decreased the relative level of miR-210 in PC-3 sphere forming cells under hypoxic condition **(Figure 19).** These data suggest that CDF modulates hypoxia-associated miRNAs in CSC-like cells of PCa.

18. Effect of CDF on the gene expression of in PDGF-D over-expressing PC-3 cells under hypoxic condition. Emerging evidence suggest that PDGF-D plays an important role in the regulation of inflammatory cytokines, CSC and EMT functions, contributing to tumor aggressiveness. In order to examine the effect of CDF on the expression of these genes in PDGF-D over-expressing PC-3 cells, we measured the levels of these mRNAs in PC-3-PDGF-D cells under hypoxic condition by real-time RT-PCR, as described above. The results show that CDF treatment decreased the relative mRNA levels of IL-6, VEGF, EZH2, Nanog, Oct4, and Vimentin, and increased the relative mRNA levels of E-cadherin in PDGF-D over-expressing PC-3 cells under hypoxic condition **(Figure 20-21). Collectively, the above results provided mechanistic role of deregulated genes under hypoxic condition that are associated with tumor aggressiveness, and that CDF was able to attenuate these molecular makers consistent with reduced tumor aggressive behaviors.**

Abstract: presented in 2012 AACR 2012 annual conference, Chicago, IL

Hypoxia has been well recognized as one of the fundamentally important features associated with solid tumors and considered to play critical roles in variety of biological events, including cell growth/proliferation, survival, angiogenesis, immunosurveillance, and tumor invasion/metastasis. Hypoxia-inducible factor (HIF) 1α, a master transcription factor for hypoxia responsive genes, plays critical roles in the adaptation of tumor cells to a hypoxic microenvironment. Tumor hypoxia and over-expression of HIF-1α have been reportedly associated with radiation therapy and chemotherapy resistance, a risk of invasion and metastasis, a poor clinical prognosis of solid tumors, which leads to higher mortality of the cancer patients. Emerging evidence suggest that hypoxia and HIF signal pathways increases the acquisition of epithelial-to-mesenchymal transition (EMT), cancer stem cell (CSC) functions, and inflammation, which contribute to radiation therapy and chemotherapy resistance. However, the detailed mechanisms by which hypoxia/HIF plays in these events are not fully understood. Here, we demonstrates that hypoxia increases the productions of VEGF and IL-6, the expression of CSC genes Nanog, Oct4, EZH2 as well as miR-21, a oncogenic miRNA, in human PCa (PC-3 and LNCap) cells. The treatment with CDF, a novel Curcumin-derived analog previously showing an anti-tumor effect in vivo, inhibits the productions of VEGF and IL-6, and the expression of Nanog, Oct4, EZH2 mRNAs, as well as miR-21 in these cells under hypoxic condition. CDF also decreases cell migration in PCa cells under hypoxic condition by wound healing assay. We also found the similar results in human pancreatic cancer cells. Taken together, these data indicates the anti-tumor effect of CDF may be, in part, associated with its inhibition of tumor hypoxic pathways. Further mechanistic studies are under investigation. Since the submission of the abstract and poster presentation, we have now provided additional mechanistic findings as presented above **(Fig-1-21).**

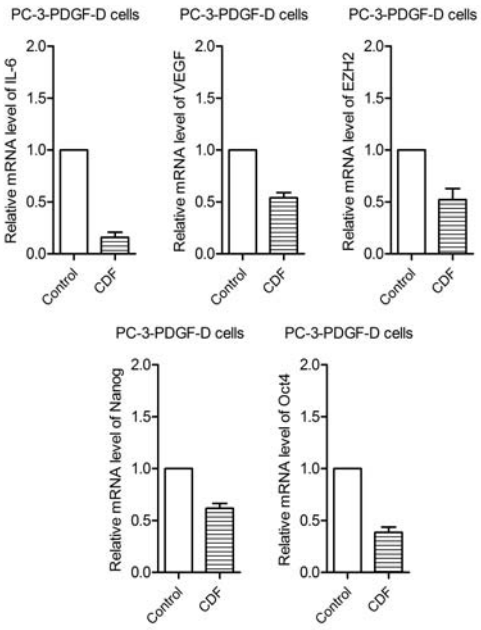

Fig-20. Effect of CDF on the relative levels of IL-6, VEGF, EZH2, Nanog, Oct4 mRNAs in PC-3-PDGF-D cells of human prostate cancer under hypoxia.

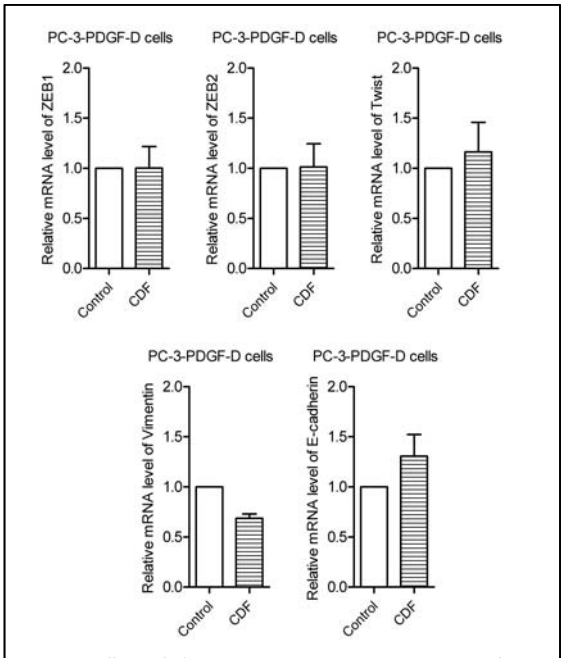

Fig-21. Effect of CDF on the relative mRNA levels of EMT markers in PC-3-PDGF-D cells of human prostate cancer under hypoxia.

Key Research Accomplishments

- **We found that hypoxia led to increased cell migration, invasion, and angiogenesis in PCa cells.**
- **Hypoxia induces the VEGF and IL-6 cytokine production in PCa cells and its CSC-like sphere forming cells.**
- **The CSC-like sphere forming cells of PCa produces a greater amount of VEGF and IL-6 cytokines under hypoxic condition, compared to its parental cells.**
- **Over-expression of HIF-1α increases the production of VEGF and IL-6 in PCa cells under hypoxic condition.**
- **Over-expression of HIF-1α and PDGF-D increases cell migration in PCa cells under hypoxic condition.**
- **Hypoxia also increases the self-renewal capacity of CSC, and the expression of CSC signature genes such as Nanog, Oct4, EZH2, CD44 and EpCAM in PCa cells and its CSC-like sphere forming cells.**
- **Hypoxia increases the expression of miR-21 and miR-210 as well as miR-21 binding to 3' UTR in PCa cells.**
- **The knock-down of miR-21 by its inhibitor down-regulates the production of VEGF and IL-6 cytokines, the formation of prostaspheres and the expression of CSC gene markers CD44, EpCAM, Nanog, Oct4, and EZH2 in PCa cells under hypoxic condition.**
- **Our novel curcumin-derived analog CDF inhibits cell survival, clonogenicity, cell migration, invasion, angiogenesis, and the self-renewal of capacity of CSC in PCa cells under hypoxic condition.**
- **CDF down-regulates HIF-1α, VEGF and IL-6 cytokine production, CSC signature genes and its products, and the expression of miR-21 and miR-210 in PCa cells under hypoxic condition.**
- **CDF increases the expression of let-7c,d and miR-101, and decreased the expression of miR-210 in the CSC-like sphere forming cells of PCa cells under hypoxic condition.**
- **CDF decreases the gene expression of mesenchymal markers of EMT phenotype ZEB1, ZEB2, Twist, and Vimentin, and increases the gene expression of epithelial marker E-cadherin in the CSC-like sphere forming cells of PCa cells under hypoxic condition.**
- **CDF inhibits VEGF and IL-6 cytokine production in HIF-1α-over-expressing PCa cells under hypoxic condition.**
- **CDF inhibits cell migration in HIF-1α- and PDGF-D-over-expressing PCa cells under hypoxic condition.**
- **CDF inhibits gene expression of VEGF, IL-6, Nanog, Oct4, and EZH2 and Vimentin, and increased the expression of E-cadherin in PDGF-D-over-expressing PCa cells under hypoxic condition.**

Reportable Outcomes

We have published two articles, and now we are preparing a new manuscript, which is being submitted to Oncogene. We also presented our data at the 103th AACR Annual Conference, Chicago, IL, 2012

Publications and manuscripts during the funding period:

Bao B, Archana Thakur, Yiwei Li, A Ahmad, A Azmi, S Banerjee, D Kong, S Ali, LG. Lum , and FH Sarkar (2012) The immunological contribution of NF-kB within the tumor microenvironment: A potential protective role of zinc as an anti-tumor agent. **BBA Reviews on Cancer** 1825:160-172. (Review)

Bao B, A Azmi, S Ali, Yiwei Li, A Ahmad, S Banerjee, D Kong, and FH Sarkar (2012) The Biological Kinship of Hypoxia with CSC and EMT and Their Relationship with Deregulated Expression of miRNAs and Tumor Aggressiveness. **BBA Reviews on Cancer** (Acceptance)

National conference and presentations during the first year of funding:

Bao B, S Ali, A Ahmad, AS. Azmi, S Banerjee, D Kong, Y Li, S Padhye, and Fazlul H. Sarkar. CDF inhibits cell migration by inactivating cancer stem cell markers, and expression of VEGF, IL-6 and miR-21 in human PCa cells under hypoxic condition. **The 103nd AACR Annual Conference**, Chicago, IL, 2012

Preparation of the manuscript being submitted to Oncogene

Bao B, A Ahmad, S Ali, A Azmi, Yiwei Li, S Banerjee, D Kong, and FH Sarkar (2012) A novel curcumin analog CD inhibit aggressive tumor phenotype by inactivation of cancer stem cell marker, and expression of VEGF, IL-6, miR-21, and miR-210 in PCa cells under hypoxic condition. **Being submitted to Oncogene**

Conclusion:

Essentially all parts of aims 1, 2 and 3 have been completed. We have published two articles, and presented the partial data at the 103th AACR Annual Conference, Chicago, IL, 2012. We are preparing a new manuscript, which is being submitted to Oncogene. During the year of funding, we found that hypoxia increases cell migration, invasion, angiogenesis, the formation of prostaspheres, increased production of VEGF and IL-6, and also increased the expression of CSC genes Nanog, Oct4, EZH2, CD44, and EpCAM as well as oncogenic miRNAs miR-21 and miR-210 in human PCa (PC-3 and LNCaP) cells. Knock-down of miR-21 by its inhibitor resulted in decreased cytokine production such as VEGF and IL-6, and decreased the formation of prostaspheres, the expression of CSC surface markers CD44 and EpCAM, and the expression of HIF-1α, VEGF, IL-6, CD44, and EpCAM in PCa cells under hypoxic condition. The treatment of cells with CDF, a novel synthetic small molecule that has been previously shown to have anti-tumor effect *in vivo*, inhibited the expression of HIF-1α, CD44, EpCAM, VEGF and IL-6, and the expression of Nanog, Oct4, EZH2 mRNAs, as well as down-regulated the expression of miR-21 and miR-210, and enhanced the expression of anti-oncogenic miRNAs let-7c,d and miR-101 in these cells under hypoxic condition. CDF also decreased cell survival, clonogenicity, migration, invasion, and self-renewal capacity of CSC in PCa cells under hypoxic condition. Taken together, these data indicates that the anti-tumor effect of CDF could in part be due to deregulation of important molecular changes that are associated with tumor hypoxic pathways.

Reference List

1 Siegel,R., Naishadham,D. and Jemal,A. Cancer statistics, 2012, CA Cancer J.Clin., *62:* 10-29, 2012.

2 Jubb,A.M., Buffa,F.M. and Harris,A.L. Assessment of tumour hypoxia for prediction of response to therapy and cancer prognosis, J.Cell Mol.Med., *14:* 18-29, 2010.

3 Moulder,J.E. and Rockwell,S. Tumor hypoxia: its impact on cancer therapy, Cancer Metastasis Rev., *5:* 313-341, 1987.

4 Sarkar,F.H., Li,Y., Wang,Z. and Kong,D. Pancreatic cancer stem cells and EMT in drug resistance and metastasis, Minerva Chir, *64:* 489-500, 2009.

5 Heddleston,J.M., Li,Z., Lathia,J.D., Bao,S., Hjelmeland,A.B. and Rich,J.N. Hypoxia inducible factors in cancer stem cells, Br.J.Cancer, *102:* 789-795, 2010.

6 Jiang,J., Tang,Y.L. and Liang,X.H. EMT: a new vision of hypoxia promoting cancer progression, Cancer Biol.Ther., *11:* 714-723, 2011.

7 McCord,A.M., Jamal,M., Shankavaram,U.T., Lang,F.F., Camphausen,K. and Tofilon,P.J. Physiologic oxygen concentration enhances the stem-like properties of CD133+ human glioblastoma cells in vitro, Mol.Cancer Res., *7:* 489-497, 2009.

8 Pietras,A., Johnsson,A.S. and Pahlman,S. The HIF-2alpha-driven pseudo-hypoxic phenotype in tumor aggressiveness, differentiation, and vascularization, Curr.Top.Microbiol.Immunol., *345:* 1-20, 2010.

9 Soeda,A., Park,M., Lee,D., Mintz,A., ndroutsellis-Theotokis,A., McKay,R.D., Engh,J., Iwama,T., Kunisada,T., Kassam,A.B., Pollack,I.F. and Park,D.M. Hypoxia promotes expansion of the CD133-positive glioma stem cells through activation of HIF-1alpha, Oncogene, *28:* 3949-3959, 2009.

10 DeSano,J.T. and Xu,L. MicroRNA regulation of cancer stem cells and therapeutic implications, AAPS.J., *11:* 682-692, 2009.

11 Perera,R.J. and Ray,A. MicroRNAs in the search for understanding human diseases, BioDrugs., *21:* 97-104, 2007.

12 Cao,P., Deng,Z., Wan,M., Huang,W., Cramer,S.D., Xu,J., Lei,M. and Sui,G. MicroRNA-101 negatively regulates Ezh2 and its expression is modulated by androgen receptor and HIF-1alpha/HIF-1beta, Mol.Cancer, *9:* 108, 2010.

13 Kulshreshtha,R., Ferracin,M., Negrini,M., Calin,G.A., Davuluri,R.V. and Ivan,M. Regulation of microRNA expression: the hypoxic component, Cell Cycle, *6:* 1426-1431, 2007.

14 Kulshreshtha,R., Davuluri,R.V., Calin,G.A. and Ivan,M. A microRNA component of the hypoxic response, Cell Death.Differ., *15:* 667-671, 2008.

15 Pocock,R. Invited review: decoding the microRNA response to hypoxia, Pflugers Arch., *461:* 307-315, 2011.

16 Pang,Y., Young,C.Y. and Yuan,H. MicroRNAs and PCa, Acta Biochim.Biophys.Sin.(Shanghai), *42:* 363-369, 2010.

17 Quero,L., Dubois,L., Lieuwes,N.G., Hennequin,C. and Lambin,P. miR-210 as a marker of chronic hypoxia, but not a therapeutic target in PCa, Radiother.Oncol., 2011.

18 Ali,S., Ahmad,A., Banerjee,S., Padhye,S., Dominiak,K., Schaffert,J.M., Wang,Z., Philip,P.A. and Sarkar,F.H. Gemcitabine sensitivity can be induced in pancreatic cancer cells through modulation of miR-200 and miR-21 expression by curcumin or its analogue CDF, Cancer Res., *70:* 3606-3617, 2010.

19 Bao,B., Ali,S., Kong,D., Sarkar,S.H., Wang,Z., Banerjee,S., Aboukameel,A., Padhye,S., Philip,P.A. and Sarkar,F.H. Anti-tumor activity of a novel compound-CDF is mediated by regulating miR-21, miR-200, and PTEN in pancreatic cancer, PLoS.One., *6:* e17850, 2011.

20 Korkaya,H., Liu,S. and Wicha,M.S. Regulation of cancer stem cells by cytokine networks: attacking cancer's inflammatory roots, Clin.Cancer Res., *17:* 6125-6129, 2011.

21 Levina,V., Marrangoni,A.M., DeMarco,R., Gorelik,E. and Lokshin,A.E. Drug-selected human lung cancer stem cells: cytokine network, tumorigenic and metastatic properties, PLoS.One., *3:* e3077, 2008.

22 Kong,D., Banerjee,S., Huang,W., Li,Y., Wang,Z., Kim,H.R. and Sarkar,F.H. Mammalian target of rapamycin repression by 3,3'-diindolylmethane inhibits invasion and angiogenesis in platelet-derived growth factor-D-overexpressing PC3 cells, Cancer Res., *68:* 1927-1934, 2008.

23 Wang,Z., Kong,D., Banerjee,S., Li,Y., Adsay,N.V., Abbruzzese,J. and Sarkar,F.H. Down-regulation of platelet-derived growth factor-D inhibits cell growth and angiogenesis through inactivation of Notch-1 and nuclear factor-kappaB signaling, Cancer Res., *67:* 11377-11385, 2007.

24 Bao,B., Ali,S., Banerjee,S., Wang,Z., Logna,F., Azmi,A.S., Kong,D., Ahmad,A., Li,Y., Padhye,S. and Sarkar,F.H. Curcumin Analogue CDF Inhibits Pancreatic Tumor Growth by Switching on Suppressor microRNAs and Attenuating EZH2 Expression, Cancer Res., *72:* 335-345, 2012.

25 Bao,B., Wang,Z., Ali,S., Ahmad,A., Azmi,A.S., Sarkar,S.H., Banerjee,S., Kong,D., Li,Y., Thakur,S. and Sarkar,F.H. Metformin Inhibits Cell Proliferation, Migration and Invasion by Attenuating CSC Function Mediated by Deregulating miRNAs in Pancreatic Cancer Cells, Cancer Prev.Res.(Phila), 2012.

26 Bao,B., Wang,Z., Ali,S., Kong,D., Banerjee,S., Ahmad,A., Li,Y., Azmi,A.S., Miele,L. and Sarkar,F.H. Over-expression of FoxM1 leads to epithelial-mesenchymal transition and cancer stem cell phenotype in pancreatic cancer cells, J.Cell Biochem., *112:* 2296-2306, 2011.

27 Bao,B., Wang,Z., Ali,S., Kong,D., Li,Y., Ahmad,A., Banerjee,S., Azmi,A.S., Miele,L. and Sarkar,F.H. Notch-1 induces epithelial-mesenchymal transition consistent with cancer stem cell phenotype in pancreatic cancer cells, Cancer Lett., *307:* 26-36, 2011.

28 Hua,Z., Lv,Q., Ye,W., Wong,C.K., Cai,G., Gu,D., Ji,Y., Zhao,C., Wang,J., Yang,B.B. and Zhang,Y. MiRNA-directed regulation of VEGF and other angiogenic factors under hypoxia, PLoS.One., *1:* e116, 2006.

29 Kulshreshtha,R., Ferracin,M., Wojcik,S.E., Garzon,R., Alder,H., gosto-Perez,F.J., Davuluri,R., Liu,C.G., Croce,C.M., Negrini,M., Calin,G.A. and Ivan,M. A microRNA signature of hypoxia, Mol.Cell Biol., *27:* 1859-1867, 2007.